Scientific Committee on Consumer Safety

SCCS

OPINION ON

Indigofera tinctoria

COLIPA n° C170

The SCCS adopted this opinion at its 16th plenary meeting

of 18 September 2012

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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ISSN 1831-4767 ISBN 978-92-79-30776-8
Doi:10.2772/88378 ND-AQ-12-026-EN-N

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

ACKNOWLEDGMENTS

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Keywords: SCCS, scientific opinion, hair dye, Indigofera tinctoria, C170, directive 76/768/ECC

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Indigofera tinctoria, 18 September 2012

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1. BACKGROUND

Submission I for Indigofera tinctoria was submitted in October 2003.

On the 23 April 2004, the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted, by written procedure, an opinion (SCCNFP/0790/04) according to which..."*a complete safety dossier on Indigofera tinctoria is required*".

According to the current submission II (2006) Indigofera tinctoria is used as a hair dye at a maximum concentration on the head of 25%.

2. TERMS OF REFERENCE

1. *Does the SCCS consider Indigofera tinctoria safe for the consumers when used as a hair dye taken into account the scientific data provided?*

2. *And/or does the SCCS recommend any further restrictions with regard to the use of Indigofera tinctoria in any hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Indigofera tinctoria (INCI)

3.1.1.2. Chemical names

Indigofera tinctoria, dried and pulverised leafs of *Indigofera tinctoria L.*

3.1.1.3. Trade names and abbreviations

Colipa C170

3.1.1.4. CAS / EC number

CAS: 84775-63-3
EC: 283-892-6

3.1.1.5. Structural formula

Complex chemical mixture

3.1.1.6. Empirical formula

Not applicable

3.1.2. Physical form

Finely divided green dispersible powder

3.1.3. Molecular weight

Molecular weight: /

3.1.4. Purity, composition and substance codes

Indigofera tinctoria batch FA 177949 was shown to contain 3.56% indican (CAS 4873-60-5, Indoxyl ß-D-glucoside). The identification was performed by NMR, LC-MS and TLC.

Water extract of Indigofera tinctoria is known to contain two major components: indigo and its isomer indirubin (Chanayath et al., 2002).

Comment
Only the content of indican in one batch of *Indigofera tinctoria* was reported. A complete chemical characterisation of *Indigofera tinctoria* was not performed.

3.1.5. Impurities / accompanying contaminants

Indigofera tinctoria batch FA 177949 was shown to contain 1.3 ppm Pb, 0.03 pm Cd and 0.01 ppm Hg. (Ref. 19)
Analysis of various pesticides at 0.05 ppm and 0.1 ppm detection limits showed that these were not present in *Indigofera tinctoria* batch FA 177949 (Ref. 21)

The ash of *Indigofera tinctoria* batch FA 177949 determined according to European Pharmacopeia 5,0,2,4,16 was 22%, and the hydrochloric acid soluble ash was 8.8%.

3.1.6. Solubility

Indigofera tinctoria as greenish powder is partly soluble in water with a varying degree of solubility of its constituents.

Comment
No details about the constituents of *Indigofera tinctoria* and their solubility were provided.

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: /

3.1.8. Additional physical and chemical specifications

Melting point:	/
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm):	/

3.1.9. Homogeneity and Stability

Indican was stable (CV max 4.4%) in a suspension of Indigofera tinctoria (Henna Schwarz) in DMSO for up to 4 hours (0, 2h, 4h) at room temperature.

Comment
Stability of indican and other possible extracted chemicals of Indigofera tinctoria in water suspension (pulp as described in 3.2 Function and Uses) was not reported.

General Comments to physico-chemical characterisation

- Only the content of indican in *Indigofera tinctoria* is reported. A complete chemical analysis of *Indigofera tinctoria* powder is required.
- *Indigofera tinctoria* of only one batch has been analysed in the submitted studies. The variation in the content of indican in various batches is not known.
- In a study (Kamal et al. 1993), six rotenoids (deguelin, dehydrodeguelin, rotenol, rotenone, tephrosin and sumatrol) were isolated, identified and quantified in Indigofera tinctoria. The content of rotenoids in *Indigofera tinctoria* was not reported in the dossier.
- Stability of indican and other constituents of *Indigofera tinctoria* in water suspension (pulp as described in 3.2 Function and Uses) was not reported.

3.2. Function and uses

The pulverised leaf of Indigofera tinctoria is used as a hair dye. A hair dye formulation is prepared by adding 300 ml boiling water to 100 g Indigofera tinctoria as dried plant powder in a non-metallic bowl. The mixture (pulp) is stirred. After cooling, the pulp is applied on the hair for a period between 15 minutes up to 2 hours. Thereafter, the aqueous Indigofera tinctoria pulp is thoroughly rinsed out with water and the hair is washed with a mild shampoo to eliminate any residues.

A representative hair dye formulation contains a maximum of 25% Indigofera tinctoria powder suspended in 75% water.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

No data submitted

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404 (1992)
Species:	New Zealand white rabbits
Group size:	3 females
Test substance:	DA 060492 (Indigofera tinctoria leaf powder)
Batch:	type 210741, 03.02.94.
Purity:	/
Dose levels:	approx. 80 mg/cm²
Route:	dermal
Exposure:	4 h
GLP:	in compliance
Date:	1994

A cellulose patch with 0.5 g of DA 060492 was spread over approx. 6 cm² and fixed with a non-irritating tape. Animals were examined for erythema/eschar and oedema as well as for local and systemic signs: 1, 24, 48 and 72 h after patch removal.

Results
All animals revealed very slight oedema and erythema (score 1) after 1 h. Only one very slight erythema remained in 1 animal after 24 h and scoring at all further time-points was negative for all parameters.

Conclusion
The study authors considered DA 060492 as non-irritating to the skin in this test.
Ref.: 2 (subm I); 10 (subm II)

Comment
DA 060492, applied 'as is' under the conditions of this test, caused mild transient irritation to rabbit skin.

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405 (1987)
Species:	New Zealand white rabbits
Group size:	3 females
Test substance:	DA 060492 (Indigofera tinctoria leaf powder)
Batch:	type 210741, 03.02.94
Purity:	/
Dose levels:	47, 73, 71 mg, applied to animals 1, 2, and 3 respectively
Route:	ocular
GLP:	in compliance
Date:	1994

DA 060492 was administered to the conjunctival sac of the right eye, and the eye was held closed for about 1 second. The animals were examined at: 1, 24, 48 and 72 h using an otoscope-lamp. The left eye served as the control.

Results
Redness and chemosis were observed in 3 animals each 1 h after exposure to DA 060492. No alterations of cornea and iris were observed at this time-point, but scoring at later time-points revealed corneal opacity in 2 animals. Redness and chemosis remained for several hours, and aggravated occasionally. Additional scoring after 6 and 8 days revealed no corneal opacity, but conjunctival redness and chemosis remained for 6 days in one animal.

Conclusion
DA 060492 is considered to be irritating to the eye.
Ref.: 3 (subm I); 11 (subm II)

3.3.3. Skin sensitisation

Maximisation (Magnusson and Kligman) test, study 1

Guideline:	Directive 92/69/EEC, method B.6. (1992)	
Species:	Hartley guinea pigs	
Strain:	Crl:(HA)BR	
Group size:	main study:	10 females (+5 controls)
	preliminary study:	3 females
Test substance:	DA 060492	
Batch:	type 210741, 03.02.94.	
Purity:	/	
Dose levels:	intradermal induction:	0.1% physiological saline
	epicutaneous induction:	40% petrolatum
	epicutaneous challenge:	40% petrolatum
GLP:	in compliance	
Date:	1994	

DA 060492, suspended in physiological saline, was applied to 10 guinea pigs in the Magnusson and Kligman "maximisation test". 1,4-phenylenediamine was used as positive control substance. Freund's complete adjuvant was used.

Results
Following challenge exposure "well defined erythema" or "severe erythema and/or oedema" were observed in all animals (10/10) after 24 h and / or 48 h. One negative control (1/5) also showed a reaction.

Conclusion
Based on these results DA 060492 is a sensitizer following skin contact in this maximisation test.

Ref.: 4 (subm I); 12 (subm II)

Maximisation (Magnusson and Kligman) test, study 2

Guideline:	OECD 406 (1992)
Species:	albino guinea pigs
Strain:	Dunkin Hartley
Group size:	20 males (10 + 10 controls)
Test substance:	DA 060492
Purity:	/
Batch:	03.02.94
Dose levels:	intradermal induction: 0.25% aqueous solution epicutaneous induction: 12.5% aqueous solution epicutaneous challenge: 12.5% aqueous solution
GLP:	in compliance
Date:	1995

DA 060492, suspended in distilled water, was applied to 10 guinea pigs in the Magnusson and Kligman "maximisation test". Freund's complete adjuvant was used. Sodium lauryl sulphate was applied to cause irritation.

Results
8 of 10 guinea pigs showed slight to intense erythema following challenge exposure after 24 h. 48 h after challenge, moderate to intense erythema were scored in 5 animals.

Conclusion
Based on these results, DA 060492 induced delayed contact hypersensitivity.

Ref.: 5 (subm I); 31 (subm II)

Buehler study

Guideline:	Directive 92/69/EEC, method B.6. (1992)
Species:	Hartley guinea pigs
Strain:	Crl:(HA)BR
Group size:	test group: 20 females
Control group:	10 females
Test substance:	DA 060492
Batch:	type: 210741, 03.02.94.
Purity:	/
Dose levels:	epicutaneous induction (3x): 20% aqueous solution epicutaneous challenge: 20% aqueous solution
GLP:	in compliance
Date:	1995

The DA 060492, suspended in distilled water, was applied to 20 guinea pigs in the Buehler sensitisation test. 1,4-phenylenediamine was used as positive control substance.

Results
No skin reactions were noted after visual examination. However, the test substance stained the skin green, which may have masked any erythema. Histopathological examination following treatment with DA 060492 showed some dermal changes in 2/20 animals.

Conclusion
The study authors considered DA 060492 as non-sensitising following skin contact in this test.

Ref.: 6 (subm I), 13 (subm II)

Comment
The green staining may have masked erythema, if present.

Human dermal sensitisation

Guideline:	/
Species:	human
Group size:	4 males and 49 females
Test substance:	indigo dispersible powder, applied as a paste
Batch:	/
GLP:	/

The original study is not available; it is reported in a review. The study does not respond to any guidelines and is judged not to be suitable for the investigation of sensitising properties. However, 3/53 subjects developed erythema on induction and 2/53 on challenge.

No report submitted. Data taken from review article.

Ref.: 1, subm I

Comment
The SCCS considers such studies are unethical.

Overall comment Sensitisation
Two Magnusson & Kligman studies show that DA 060492 is a contact allergen in this model and may be considered a strong contact allergen.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428
Species/strain:	Porcine ear skin 400 µm thick
Replicates:	6 in each of 3 experiments
Skin integrity:	conductivity
Chambers:	Glass diffusion cells; diameter 1.135 cm
Test substance:	Indigofera tinctoria (Henna Schwarz)
Batch:	FA177949
Purity	(containing 3.56% Indoxyl β-D-glucoside)
Reference substance:	Indican (Indoxyl β-D-glucoside)
Batch:	015K1190
Dose level:	20 µl/cm² (corresponding to 157 ± 4.8 µg/cm² Indican (Indoxyl β-D-glucoside))
Purity:	99%
Receptor fluid:	physiological saline
Solubility in receptor:	/
Stability in receptor:	stable for duration of experiment
Method of Analysis:	HPLC; LOD 0.6ng/ml in saline; LOQ 1.25ng/ml in saline

GLP: compliant
Study period: 2006

The main active ingredient and therefore, the relevant component of *Indigofera tinctoria* (Henna Schwarz) is Indoxyl β-D-glucoside, which was investigated for its dermal absorption on porcine skin.

Three independent experiments were performed with 6 diffusion cells per experiment but only the first and third series were considered since no results for integrity measurements of the skin were obtained in the second experiment.

The study was performed on fresh dermatomed pig skin samples that were used on the day of slaughter and mounted on diffusion cells between donor and receptor chambers.

In each experiment 6 replicates of the formulation were analysed. 20 µl of the formulation was applied to each membrane for 30 minutes and then washed off using 2 x 1 ml deionized water, 3 x 1 ml 10% shampoo solution, and 2 x 1 ml deionized water. The penetration was determined after 24 hours under non-occluded conditions. Physiological saline was slowly pumped through the receptor chambers with a flow rate of 0.5 to 2 ml per hour. The receptor fluid was fractionated after 0.5, 1, 2, 4, 6, 8 and 24 hours after the application of the test item. After 24 hours the donor chambers were filled with 1 ml receptor solution to re-analyzed the conductivity.

The stratum corneum was separated by tape stripping from the remaining dermis and epidermis. The tape strips (10 strips per sample) were pooled and extracted for analysis. The samples were analyzed by HPLC for the presence of Indoxyl β-D-glucoside.

Results
Indican (Indoxyl β-D-glucoside) was detected in all samples relevant for dermal absorption, i.e. in the skin extracts and in the receptor fluid samples after 24 hours.

Summary of Experiments 1-2 for Indoxyl β-D-glucoside

	Experiment 1						Experiment 3					
Sample[1]	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
0.5 hours	0.414	0.384	6.24	5.35	0.312	0.384	0.420	0.384	0.775	0.763	0.348	0.348
1 hours	3.90	3.29	93.7	57.0	0.88	9.59	0.44	0.40	8.40	0.38	4.11	79.3
2 hours	25.4	23.4	274	179	13.8	53.7	5.52	0.80	35.4	24.8	14.1	9124
4 hours	63.5	68.7	378	185	25.4	97.7	127	8.75	56.5	35.0	26.1	12858
6 hours	35.3	43.5	208	175	10.1	30.9	29.8	3.28	35.2	26.8	32.6	8069
8 hours	18.5	24.5	101.1	85.1	8.56	21.3	23.7	3.14	23.5	17.3	23.3	4266
24 hours	23.7	31.0	24.1	10.8	21.9	50.8	87.9	23.50	71.2	27.9	37.8	1064
SN[2]	307	363	560	413	342	362	415	470	782	458	727	485
Wash Sol[3]	148436	144981	146359	155493	145811	141894	134735	131332	140296	145299	140435	99895
Strip Sol[4]	107	73.9	35.1	75.1	148	79.3	136	186	258	101	129	94.2
Skin extract[5]	346	937	779	557	583	866	584	318	484	798	284	470
PWL[6]	5206	10399	12830	5103	14754	22939	7576	8348	6311	5379	7540	10233
Amount pipetted (ng)	169157	169157	169157	169157	169157	169157	163770	163770	163770	163770	163770	163770
Sum Receptor Fluid (ng)[7]	171	195	1085	698	81.1	264	275	40.2	231	133	138	35460
Amount applied (ng)[8]	163951	158759	156328	164054	154403	146218	156193	155422	157458	158391	156229	153537
Amount measured, recovery (ng)[9]	149367	146549	148819	157235	146966	143466	136144	132346	142052	146790	141713	136405
Dermal absorption (ng)[10]	517	1131	1864	1254	664	1131	859	358	715	931	422	35930

1) sampling of the receptor chamber after the indicated time; values in ng
2) SN (supernatant): Indoxyl β-D-glucoside concentration of the receptor solution used for measuring the conductivity after the last sampling
3) Wash solution: after the treatment of the skin with 20 µL test item the donor chamber was washed with 2x1mL deionised water, 3x1 mL washing liquid, and 2x1 mL deionised water
4) Stripping solution: concentration of Indoxyl β-D-glucoside in the extract solution of the strips (adsorption)
5) Skin extract: concentration of Indoxyl β-D-glucoside in the extract solution of the strips (absorption)
6) PWL: amount of Indoxyl β-D-glucoside remaining in the pipette tip; this value is not considered as applied and is only needed for calculation of the mass balance.
7) Total amount of Indoxyl β-D-glucoside found in the receptor solution (penetration)
8) Amount applied: amount of Indoxyl β-D-glucoside corresponding to 20 µL of the test item applied on the skin excluding the residue in the pipette tip
9) Amount measured: the sum of the amount of Indoxyl β-D-glucoside measured in all samples except the PWL
10) Dermal absorption: the sum of the amount of Indoxyl β-D-glucoside found in the receptor fluids and in the skin extracts

Results of the shaded chamber are not used for calculation of mean dermal absorption since this value is regarded as an outlier.

One of the chambers from experiment 3 was an outlier and not used for further analysis.

Amount of Indican (Indoxyl β-D-glucoside)	Expressed as µg/cm² of skin surface mean ± S.D. (n = 11)	Expressed as % of dose mean ± S.D. (n = 11)
Receptor fluid	0.301 ±0.313	0.191 ±0.198
Stratum corneum (isolated by tape stripping)	0.121 ±0.062	0.077 ± 0.040
Epidermis + Upper dermis (after 24 hours)	0.594 ±0.227	0.380 ±0.150
Washing solution (after 30 minutes)	143 ±6.58	*91.2* ± 3.734
Total balance (recovery)	145 ±6.72	92.2 ±3.857
Dermal absorption	0.895 ±0.439	0.571 ±0.281

Conclusion
Under the reported conditions, the dermal absorption of Indoxy ß-D-glucoside as main active ingredient of Indigofera tinctoria (Henna Schwarz) was shown to be 0.90 ± 0.44 µg/cm² or 0.57 ± 0.28 % using freshly dermatomed pig skin samples.

Ref.: 26 (Subm II)

Comment
These were well performed experiments. Therefore, the mean+1SD may be used in calculating the MOS. (1.34 µg/cm² or 0.85% of the applied dose of Indoxy ß-D-glucoside).

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (30 days) oral toxicity

No data submitted

3.3.5.2. Sub-chronic (90 days) toxicity (oral, dermal)

No data submitted

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Taken from SCCNFP/0790/04, modified

Bacterial Reverse Mutation Assay

Guideline: /
Species/Strain: *Salmonella typhimurium* TA98, TA100, TA1535 and TA1538
Replicates: duplicate cultures
Test substance: Natural Indigo
Batch: /
Purity: /

Vehicle:	DMSO	
Concentration:	experiment 1:	0, 10, 100, 250, 500, 1000, 1500, 2000, 2500 and 3000 µg/plate, without and with S9-mix
	experiment 2:	0, 100, 500, 1000 and 2500 µg/plate, with S9-mix
metabolic activation:	experiment 1:	0, 25, 50 and 100 µl/plate
	experiment 2:	25 µl/plate
Treatment:	/	
GLP:	/	
Study date:	publication from 1984	

Natural Indigo was tested in strains of *Salmonella typhimurium* TA98, TA100, TA1535 and TA1538 in the presence and absence of a metabolic activation system. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn. Negative and positive controls were included.

Results
The plates incubated with Natural Indigo in the presence of S9-mix at concentrations > 3000 µg/plate showed toxic effect observed as a reduction in the number of revertants and a clearing of the bacterial background lawn.
In the absence of S9-mix a biologically relevant increase in the number of revertant colonies was not observed in any of the strains used. In the presence of S9-mix a concentration-dependent and statistically significant increase in the number of revertant colonies was observed in strains TA98 and TA1538. The best results were found with the lowest volume of S9-mix.

Conclusion
Under the experimental conditions used Natural Indigo was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 11 (subm I)

Comment
The test was not performed according to the OECD guideline 471 and not in compliance with GLP. Purity and batch number are not reported. The value of this test is limited.

Bacterial Reverse Mutation Assay

Guideline:	/	
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	
Replicates:	triplicates in two independent experiments	
Test substance:	DA 060492	
Batch:	19556	
Purity:	/	
Vehicle:	DMSO	
Concentration:	experiment 1:	1, 10, 100, 1000 and 5000 µg/plate, without and with S9-mix
	experiment 2:	30, 100, 300, 1000 and 3000 µg/plate, without and with S9-mix
Treatment:	direct plate incorporation	
GLP:	/	
Study date:	report from 22 November 1993	

DA 060492 was tested in strains of *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 in the presence and absence of a metabolic activation system. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. DA 060492 was extracted from powder of Indigo leaves ("Pflanzenpulver") in

various ways often with additional extraction with ethanol. The direct plate incorporation method was used. Negative and positive controls were included.

Results
It was impossible to completely dissolve DA 060492 in DMSO; un-dissolved rests (precipitation??) of DA 060492 were always present at the higher concentrations. Biologically relevant increases in the number of revertants were not observed for TA1535, TA1537 and TA1538. For the remaining TA98 and TA100 occasionally positive findings were found. However, these findings were due to a very low background value found for the concurrent untreated controls. Moreover, they were not reproducible and not concentration-dependently related. Consequently, these positive findings were considered not biologically relevant.

Conclusion
Under the experimental conditions used DA 060492 was not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 5 (9, subm I)

Comment
The test was not performed according to the OECD guideline 471 and not in compliance with GLP. Purity and batch number are not reported. The value of this test is limited.

Bacterial Reverse Mutation Assay

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537
Replicates:	triplicates in 2 independent experiments
Test substance:	*Indigofera tinctoria* (Henna Schwarz)
Batch:	FA 177949
Purity:	composed as a powder of dried leaves. Content of the main active ingredient Indican (indoxyl-β-D-glucosid): 3.56% (HPLC) respectively 2.01% after autoclaving.
Vehicle:	DMSO
Concentration:	0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 g/plate, without and with S9-mix
Treatment:	experiment 1: direct plate incorporation with at least 48 h incubation, without and with S9-mix experiment 2: pre-incubation method with 60 minutes pre-incubation and at least 48 h incubation, without and with S9-mix
GLP:	in compliance
Study date:	20 December 2005 – 24 April 2006

Indigofera tinctoria was tested in strains of *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537 in the presence and in the absence of a metabolic activation system. Due to microbiological contamination the test item was autoclaved (25 min at 121 °C, 2.5 bar) before use. Liver S9-fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test with all strains and 8 test concentrations up to the prescribed maximum concentration of 5000 µg/plate measuring toxicity and mutagenicity. Toxicity was measured as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn. This pre-experiment is reported as experiment 1 since evaluable plates at 5 concentrations were available for all strains used. In experiment 1 the direct plate incorporation method and in experiment 2 the pre-incubation (60 minutes) method was used. Negative and positive controls were in accordance with the OECD guideline.

Results

Precipitation of *Indigofera tinctoria* in the overlay agar was observed at 1000 g/plate and above in the test tubes and at 100 g/plate and above on the plates. The undissolved particles of *Indigofera tinctoria* had no influence on the data recording.
The plates incubated with *Indigofera tinctoria* showed normal background growth up to 5000 g/plate without and with S9-mix in all strains used. No toxic effects evident as a reduction in the number of revertants occurred in the test groups without and with S9-mix.
A biologically relevant increase in the number of revertants in any of the 5 tester strains was not observed following treatment with *Indigofera tinctoria* at any concentration level, neither in the absence or presence of S9-mix.

Conclusion
Under the experimental conditions used *Indigofera tinctoria* was not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 23

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)	
Species/strain:	Mouse lymphoma L5178Y $tk^{+/-}$ cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	19556 Indigoblätter (water extract at 70°C)	
Batch:	V 106 447	
Purity:	not applicable	
Vehicle:	distilled water	
Concentrations:	experiment 1:	0, 5, 10, 25, 50, 100, 250, 500, 1000 and 2500 µg/ml without S9-mix 0, 5, 10, 25, 50, 100, 250, 500 and 1000 µg/ml with S9-mix
	experiment 2:	25, 50, 100, 259, 500, 1000, 2000 and 3000 µg/ml with S9-mix
Treatment	4 h treatment both without and with S9-mix; expression period 72 h and a selection period of 11-14 days.	
GLP:	in compliance	
Study date:	2 May 2001 – 7 February 2002	

The test item, 19556 Indigoblätter, was assayed for mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. The test item was prepared by pouring distilled water (70°C) over the test item (1 part test item:5 parts *aqua distillata*) and by stirring them with a glass stick until it got a homogeneous paste. After 30 min without heating the paste was filtered using a cellulose filter. This filtrate was used in the experiments in a concentration relating to 5 mg/ml of the original test item. Liver S9-fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system.
Test concentrations were based on the results of a pre-test for toxicity with 5 concentrations up to 5000 µg/ml without metabolic activation measuring relative suspension growth. In the main test, cells were treated for 4 h followed by an expression period of 72 h, to fix the DNA damage into a stable *tk* mutation. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. An increase in small colonies indicated by a low large/small colonies ratio (<4) is associated with clastogenic effects and/or chromosomal aberrations. Toxicity was measured as total growth relative to the growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment without metabolic activation toxic effects were seen at concentrations higher than 1000 µg/ml. Therefore, initially with metabolic activation 1000 µg/ml and

without metabolic activation 2500 µg/ml were selected as the highest concentrations. Since in experiment 1 cytotoxicity did not reach the prescribed level of 10-20% survival, in the second experiment with S9-mix higher concentrations were used.
A biologically relevant increase in mutant frequency was not observed in the groups treated with the test item both without and with S9-mix as compared to the concurrent controls. Without S9-mix occasionally higher mutant frequencies were found. However, these higher values were due to the relative low value of the negative control compared to the test item. All values found were well within the range of the historical control data and a concentration response relationship was not observed.
An increased formation of small colonies was not found both without or with S9-mix compared to the negative controls and to the historical control data.

Conclusion
Under the experimental conditions used, 19556 Indigoblätter was not genotoxic (mutagenic and clastogenic) in this gene mutation assay in mouse lymphoma cells.

Ref.: 3 (subm I)

In Vitro Mammalian Chromosomal Aberration Test

Guideline:	OECD 473
Species/strain:	CHO-K1-BH(4) cells
Replicates:	duplicate cultures per concentration
Test substance:	DA 060492
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentrations:	0, 125, 250 and 500 µg/ml without and with S9-mix
Treatment	18 h and 28 h treatment without S9-mix; harvest time immediately after the end of treatment 3 h treatment with S9-mix; harvest time 18 and 28 h after start of treatment
GLP:	in compliance
Study date:	11 January 1995 – 8 May 1995

DA 060492 has been investigated for the induction of chromosomal aberrations in CHO- K1-BH(4) cells both in the absence and presence of metabolic activation. Liver S9-fraction from rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a preliminary cytotoxicity assay measuring cell morphology and mitotic index with concentrations up to 500 µg DA 060492/ml. Cytotoxicity was determined 18 and 28 h after start of treatment.
In the main test, cells were treated for 3 h (with S9-mix) or 18 and 28 h (without S9-mix) and harvested 18 and 28 h after the start of treatment. Approximately 2 h before harvest, each culture was treated with colcemid (0.2 µg/ml culture medium) to block cells at metaphase of mitosis. Chromosome (metaphase) preparations were stained with 2% Giemsa and examined microscopically for chromosomal aberrations and the mitotic index. Reduction in the mitotic index was taken as a measure for cytotoxicity. Negative and positive controls were in accordance with the OECD guideline.

Results
In the main test the required reduction in mitotic index of 50% was only reached in the groups without S9-mix and a harvest time of 18 h.
Both in the absence and presence of S9-mix, a biologically relevant increase in cells with chromosome aberrations was not found for any time point at both harvest times.

Conclusion

Under the experimental conditions used, DA 060492 was not genotoxic (clastogenic) in the chromosome aberration test in CHO cells both in the absence and the presence of S9 metabolic activation.

Ref.: 9 (17, subm I)

Comment
As the required reduction in mitotic index of 50% was only reached in 1 of the 4 treatment groups, the test is of limited relevance.

In Vitro Mammalian Chromosomal Aberration Test

Guideline:	OECD 473 (1997)	
Species/strain:	V79 cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	*Indigofera tinctoria* (Henna Schwarz)	
Batch:	FA 177949	
Purity:	composed as a powder of dried leaves. Content of the main active ingredient Indican (indoxyl-β-D-glucosid): 3.56% (HPLC) respectively 2.01% after autoclaving.	
Vehicle:	DMSO	
Concentrations:	experiment 1:	0, 62.5, 125 and 250 µg/ml without and with S9-mix
	experiment 2:	0, 31.3, 62.5 and 125 µg/ml without S9-mix 0, 125, 250 and 500 µg/ml with S9-mix
Treatment	experiment 1:	4 h treatment without and with S9-mix; harvest time 18 h after the start of treatment
	experiment 2:	4 h treatment with S9-mix; harvest time 28 h after start of treatment. 18 and 28 h (250 µg/ml only) treatment without S9-mix; harvest time 18 h and 28 h after start of treatment
GLP:	in compliance	
Study date:	23 November 2005 – 3 May 2006	

Indigofera tinctoria has been investigated for the induction of chromosomal aberrations in V79 cells both in the absence and presence of metabolic activation. The cells were tested on stock cultures for mycoplasma contamination and karyotype stability, measured as chromosome number. Liver S9-fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test on cell growth inhibition in order to determine the cytotoxicity of the test item. The experimental conditions in this pre-test were identical to those of the main test. A quantitative evaluation of cell number and morphology was made 4 h and 24 h after the start of treatment.
In the main test, cells were treated for 4 h (without and with S9-mix) or 18 h and 28 h (without S9-mix) and harvested 18 h and 28 h after the start of treatment. Approximately 2.5 h before harvest, each culture was treated with colcemid (0.2 µg/ml culture medium) to block cells at metaphase of mitosis. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations, polyploidy and the mitotic index. Reduction in the mitotic index was taken as a measure for cytotoxicity. For evaluation of cytotoxicity 2 additional cultures per concentration not treated with colcemid were set up in parallel. These cultures were stained after 18 or 28 h and evaluated for a putative reduction in cell number which is a measure for cytotoxicity. Negative and positive controls were in accordance with the OECD guideline.

Results

In the pre-test precipitation seen as grinded plant fibres was observed at 7.8 µg/ml and above. In the main test precipitation occurred at 62.5 µg/ml and above except for the 28 h harvest group where precipitation was observed at 250 µg/ml.
In experiment 2 in the presence of S9-mix clear cytotoxicity evident by reduced cell numbers, was found at the highest concentration scored. In all other groups, cultures showing clear cytotoxicity were not scorable for chromosome aberrations, due to a reduced cell number or low metaphase numbers and/or metaphase quality.
At a harvest time of 18 h a biologically relevant increase in the number of cells with chromosomal aberrations was not found at any of the concentrations evaluated, both without and with S9-mix. In contrast, in experiment 2 at a 28 h harvest, a biologically and statistically significant increase in the number of cells with chromosome aberrations was found in the absence of S9-mix (only 250 µg/ml tested); with S9-mix the increase in the number of cells with chromosome aberrations was concentration-dependent. In both experiments, an increase in polyploid cells was not observed.

Conclusion
Under the experimental conditions used, *Indigofera tinctoria* was genotoxic (clastogenic) in the chromosome aberration test in V79 cells.

Ref: 24

Comment
In most of the treated groups, the cultures that showed the required cytotoxicity were not scorable for chromosome aberrations, due to a reduced cell number or low metaphase numbers and/or metaphase quality.

In Vitro **Mammalian Chromosomal Aberration Test**

Guideline:	OECD 473 (1997)	
Species/strain:	V79 cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	*Indigofera tinctoria* (Henna Schwarz)	
Batch:	FA 177949	
Purity:	composed as a powder of dried leaves. Content of the main active ingredient Indican (indoxyl-β-D-glucosid): 3.56% (HPLC) respectively 2.01% after autoclaving.	
Vehicle:	DMSO	
Concentrations:	experiment 1:	0, 250, 500 and 1000 µg/ml without and with S9-mix
	experiment 2:	0, 62.5, 125 and 250 µg/ml without S9-mix 0, 31.3, 62.5 and 125 µg/ml with S9-mix
Treatment	experiment 1:	4 h treatment without and with S9-mix; harvest time 18 h after the start of treatment
	experiment 2:	4 h treatment with S9-mix; harvest time 28 h after start of treatment. 18 and 28 h (250 µg/ml only) treatment without S9-mix; harvest time 18 h and 28 h after start of treatment.
GLP:	in compliance	
Study date:	15 March 2006 – 18 July 2006	

Indigofera tinctoria has been investigated for the induction of chromosomal aberrations in V79 cells both in the absence and presence of metabolic activation. The cells were tested on stock cultures for mycoplasma contamination and karyotype stability, measured as chromosome number. Liver S9-fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test on cell growth inhibition in order to determine the cytotoxicity of the test item. The experimental conditions in this pre-test were identical to those of the main

test. A quantitative evaluation of cell number and morphology was made 4 h and 24 h after the start of treatment.
In the main test, cells were treated for 4 h (without and with S9-mix) or 18 h and 28 h (without S9-mix) and harvested 18 h and 28 h after the start of treatment. Approximately 2.5 h before harvest, each culture was treated with colcemid (0.2 µg/ml culture medium) to block cells at metaphase of mitosis. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations, polyploidy and the mitotic index. Reduction in the mitotic index was taken as a measure for cytotoxicity. For evaluation of cytotoxicity 2 additional cultures per concentration not treated with colcemid were set up in parallel. These cultures were stained after 18 or 28 h and evaluated for a putative reduction in cell number which is a measure for cytotoxicity. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test precipitation seen as grinded plant fibres was observed at 7.8 µg/ml and above. In the main test precipitation occurred at all concentrations even in the lowest ones applied.
In both experiments, no toxic effects indicated by reduced cell numbers or reduced mitotic indices below 50% of the controls were observed. However, in experiment 2 in the absence of S9-mix, the 2 highest concentrations and in the presence of S9-mix the 3 highest concentrations were not evaluable for chromosome aberrations due to the low metaphase number and precipitation of *Indigofera tinctoria* on the slides.
A biologically relevant increase in the number of cells with chromosomal aberrations was not found at any of the concentrations evaluated without and with S9-mix in both experiments. An increase in polyploid cells was not observed as well.

Conclusion
Under the experimental conditions used, *Indigofera tinctoria* was not genotoxic (clastogenic) in the chromosome aberration test in V79 cells.

Ref: 28

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Taken from SCCNFP/0790/04 but modified

***In vivo* Mammalian Erythrocytes Micronucleus Test**

Guideline:	/
Species/strain:	Swiss mice CD1
Group size:	10 mice/dose group
Test substance:	Natural Indigo
Batch:	/
Purity:	/
Vehicle:	aqueous solution of 10% Arabic gum
Dose level:	0, 0.1, 0.5, 1 and 2 g/kg bw
Route:	gastric tubage, 2 administration 24 h apart
Sacrifice times:	30 and 54 h after the first administration
GLP:	/
Study date:	publication from 1984

Natural Indigo was investigated for the induction of micronuclei in bone marrow cells of mice. Mice were treated by gavage twice 24 h apart with 0, 0.1, 0.5, 1 and 2 g/kg bw. Toxicity and thus exposure was not determined. Bone marrow preparations were stained with May-Grunwald/Giemsa and examined microscopically for micronuclei. Negative and positive controls were included.

Results

A biologically relevant increase in the percentages of PCEs containing MN was not observed. No information is presented about the presence of the test item in the target cells.

Conclusion
Under the experimental conditions used Natural Indigo did not induce a biologically relevant increase in the number of erythrocytes with micronuclei of treated mice and, consequently, Natural Indigo is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.
Ref.: 11 (subm I)

Comment
The study was not performed under GLP compliance and not according to the OECD guideline. Purity and batch number were not reported. Indications that bone marrow cells were exposed were lacking. Consequently, the value of this test is limited.

In vivo Mammalian Erythrocytes Micronucleus Test

Guideline:	OECD 474 (1997)
Species/strain:	NMRI mice
Group size:	5 mice/sex/group
Test substance:	19556 Indigoblätter
Batch:	V 105 447
Purity:	/
Vehicle:	distilled water
Dose level:	0, 200, 1000 and 2000 mg/kg bw
Route:	i.p. injection
Sacrifice times:	24 and 48 h (highest dose only) after injection
GLP:	in compliance
Study date:	21 March 2001 – 23 January 2003

19556 Indigoblätter was investigated for the induction of micronuclei in bone marrow cells of mice. The test item was prepared by pouring distilled water (70°C) over the test item (1 part test item:5 parts aqua distellata) and by stirring them with a glass stick until it got a homogeneous paste. After 30 min without heating the paste was filtered using a cellulose filter. This filtrate was used in the experiments in a concentration relating to 200 mg/ml of the original test item. 10 mg/kg bw of the filtrate corresponds to a dose of 2000 mg/kg bw. Test concentrations were based on the results of a pre-test for toxicity. Mice were treated ip with 2000 mg/kg bw and observed for mortality and pharmacological signs up to 48 h after dosing.
In the micronucleus test mice were treated by ip injection with 0, 200, 1000 and 2000 mg/kg bw. The mice were examined for mortality and pharmacological signs after dosing. Bone marrow cells were collected 24 h and 48 h (highest dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Bone marrow preparations were stained with May-Grunwald/Giemsa and examined microscopically for the NCE/TE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test for toxicity, mice treated with 2000 mg/kg bw showed systemic toxic effects, *i.e.* lethargy at 6, 24 and 48 h after dosing. Due to this result 2000 mg/kg bw was chosen as the top dose. In the main test no signs of toxicity were found within 24 h after dosing. However, after 48 h 3 male mice died 1-2 h before bone marrow preparation whereas 1 male and 1 female mouse showed lethargy.
The bone marrow of the 3 dead mice was collected immediately after compound-related death. Due to the process of erythropoiesis *in vivo* and the mechanism of micronucleus formation in the polychromatic erythrocytes false positive or false negative results can be excluded. All other bone marrow preparations were performed according to the guideline.

The ratio PCE/TE decreased after treatment with 19556 Indigoblätter both in male and female mice. In female mice this decrease was dose-related.
In comparison to the concurrent vehicle controls, there was no biologically relevant or statistically significant increase in the number of erythrocytes with micronuclei at any preparation interval and dose level of 19556 Indigoblätter. All values found were within the range of the historical control data.

Conclusion
Under the experimental conditions used 19556 Indigoblätter did not induce a biologically relevant increase in the number of erythrocytes with micronuclei of treated mice and, consequently, 19556 Indigoblätter is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 4 (19, subm I)

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

No data submitted

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

In a review, the traditional Chinese medicine Dang Gui Lu Hui pills, a mixture of 11 Chinese medical herbs including *Indigofera tinctoria,* was used to treat chronic myelocytic leukemia. Indigorubin was found to be the active extract of *Indigofera tinctoria* for these therapeutic effects.

In India and China, according to Hagers Handbuch der pharmazeutischen Praxis, *Indigofera tinctoria* is used for the treatment of tumours as well as extracts for the therapy of epilepsy, nervous impairments, bronchitis and external for the treatment of wounds. It is also used in homeopathy.

The applicant stated that the validity and reliability could not be proven since no details were provided.

(Ref: 7, 6 (subm II))

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Not applicable

3.3.14. Discussion

Physico-chemical properties
The pulverised leaf of *Indigofera tinctoria* is used as a hair dye. A representative hair dye formulation contains a maximum of 25% Indigofera tinctoria powder suspended in 75% water. Indigofera tinctoria of only batch has been analysed in the submitted studies. The variation in the content of indican in various batches is not known. A complete chemical characterization of Indigofera tinctoria powder has not been performed, only the characterization and quantification of indoxyl beta-D-glucoside (indican) in Indigofera tinctoria has been performed. In a study, six rotenoid (deguelin, dehydrodeguelin, rotenol, rotenone, tephrosin and sumatrol were isolated, identified and quantified in Indigofera tinctoria. The content of rotenoids in Indigofera tinctoria was not reported in the dossier. A complete chemical analysis of Indigofera tinctoria powder is required. Stability of indican and other constituents of Indigofera tinctoria in water suspension (pulp as described in 3.2 Function and Uses) was not reported.
Water extract of Indigofera tinctoria is known to contain two major components: indigo and its isomer indirubin (Chanayath et al., 2002).

Irritation, sensitisation
DA 060492, applied 'as is' under the conditions of this test, caused transient irritation to rabbit skin. DA 060492 caused irritation to the rabbit eye.
Two Magnusson & Kligman studies show that DA 060492 is a contact allergen in this model and may be considered a strong contact allergen.

Dermal absorption
The main active ingredient and therefore, the relevant component of *Indigofera tinctoria* (Henna Schwarz) is Indoxyl β-D-glucoside, which was investigated for its dermal absorption on porcine skin. *Indigofera tinctoria* (Henna Schwarz) containing 3.56% Indoxyl β-D-glucoside was used. The dose was 20 µl/cm² (corresponding to 157 ± 4.8 µg/cm² (Indoxyl β-D-glucoside). Under the reported conditions, the dermal absorption of Indoxy ß-D-glucoside as main active ingredient of Indigofera tinctoria (Henna Schwarz) was shown to be 0.90 ± 0.44 µg/cm² or 0.57 ± 0.28% using freshly dermatomed pig skin samples.

General toxicity
The toxicological data is derived from 'Toxicology of indigo, a review (1987). This review provided no systemic toxicological data on *Indigofera tinctoria* leaf powder but was based on sparse description of studies of synthetic *Indigofera tinctoria D & C Blue no 6* that was also inadequate to assess the systemic toxicity. Some information was provided of medicinal use of *Indigofera tinctoria* in traditional Chinese and Indian medicine and also in homeopathy, but even the applicant considered that the validity and reliability of these therapies could not be proven.

Mutagenicity
Overall, the genotoxicity of *Indigofera tinctoria* is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome

aberrations and aneuploidy. Under *in vitro* conditions *Indigofera tinctoria* did generally not induce gene mutations in bacteria nor in mammalian cells. In one gene mutation test in bacteria with limited value due to a bad performance of the test an induction of the number of revertants was observed. This positive finding was considered not biologically relevant. *Indigofera tinctoria* induced an increase in the number of cells with chromosome aberrations in one chromosome aberration test with limited value whereas in a well performed test it was not genotoxic.
The positive finding in the single *in vitro* chromosome aberration test was not confirmed in a well performed *in vivo* experiment. *Indigofera tinctoria* exposure of mice did not result in an increase in erythrocytes with micronuclei.
Consequently based on the present reports *Indigofera tinctoria* can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data submitted

Comparison between the toxicology of leaf extract of Indigofera tinctoria and synthetic indigo (95% pure) is not feasible. The sole chemical information provided in this dossier is that the aqueous leaf extract of Indigofera tinctoria contains ~3% of the glycoside indican that hydrolyses to the aglycone, indigo and β-D-glucose. A complete chemical analysis of Indigofera tinctoria powder and knowledge of exposure from all of its constituents is required.
As there was no NOAEL, the applicant proposed back-calculations for a MOS of 100 (NOAEL/SED), assuming NOAEL of 0.87 mg/kg bw or a MOS of 1000 if the NOAEL was 8.7 mg/kg bw using the systemic exposure dose (SED) of 0.0087 mg/kg bw. This SED was based on the percutaneous absorption of Indican (Indoxyl β-D-glucoside) through dermatomed pig skin in vitro. The applicant considered such low postulated NOAELs were unlikely, if compared with other plant powders (e.g. NOAEL of Lawsonia inermis = 40 mg/kg bw from a 90 day oral rat study). An additional argument was that they considered the NOAEL would be much higher, especially for a plant powder used in traditional Chinese medicine.

The SCCS considered the argumentation, but felt extrapolation from such a poor knowledge base is invalid.

4. CONCLUSION

The SCCS is of the opinion that the safety of *Indigofera tinctoria* used as a hair dye at a maximum concentration on the head of 25% cannot be assessed due to incomplete information.

The safety of Indigo (CI 73 000) used as a colorant should be re-assessed.

5. MINORITY OPINION

Not applicable

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Taken from SCCNFP/0790/04

Annex

Toxicological Evaluation and Characterisation on synthetic indigo, CI 73000

1.1. General

Synthetic indigo is listed as CI 73000 in Annex IV, part 1 – list of colouring agents allowed for use in cosmetic products – to Directive 76/768/EEC on cosmetic products; field of application 1: colouring agents allowed in all cosmetic products.

It was first produced in 1897.

1.1.1. Primary name

CI 73000 (INCI) is synthetic indigo

1.1.2. Chemical names

2-(1,3-dihydro-3-oxo-2H-indol-2ylidene)-1,2-dihydro-3H-indol-3-one
Pigment Blue 66, Vat Blue 1, indigo

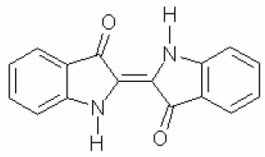
1.1.3. Trade names and abbreviations

D&C Blue n° 6
COLIPA n° C170

1.1.4. CAS / EC / Colour Index number

CAS:	482-89-3
EC:	207-586-9
Colour Index:	CI 73000

1.1.5. Structural formula



1.1.6. Empirical formula

$C_{18}H_{10}N_2O_2$

Mol weight: 262.3 g/mol

1.1.7. Purity, composition and substance codes

Specification of FDA certified D&C Blue No.6 (recrystallised synthetic dye), 99% (colour content):

Total colour content:	> 95%
Volatile matter (135°C):	< 3%
Insoluble matter in N,N'-dimethylformamide:	< 1%
Isatin:	< 0.3%
Anthranilic acid:	< 0.3%
Indirubin:	< 1%
Pb:	10 ppm
As:	3 ppm
Hg:	1 ppm

1.1.8. Physical properties

Appearance:	Dark blue powder
Melting point:	Sublimation at approx. 300°C, decomposition at 390°C
Boiling point:	/
Density:	ca. 0.56 g/cm³
Rel. vap. dens.:	/
Vapour Press.:	/
Log Pow:	3.69

1.1.9. Solubility

Water: /
Ethanol: /

1.1.10 Stability

Stable at room temperature when stored in dark. (No time limit available)

General comments on analytical and physico-chemical characterisation

* FDA certificate of the test material(s) used in the experiments is not available.
* purity of the "textile grade" indigo used in some experiments is not reported.

1.2. Function and uses

CI 73000 is permitted for use in all types of cosmetic products. It is also used for dyeing fabrics.

TOXICOLOGICAL CHARACTERISATION

1.3. Toxicity

1.3.1. Acute oral toxicity

Species/strain:	Rat, Sprague Dawley
Group size:	5 males per group
Test substance:	"Certified D&C Blue No.6"
Dose:	31.6, 100, 316, 1000 and 3160 mg/kg bw
Vehicle:	0.5% CMC aqueous solution
Results:	no mortalities, LD50orl> 3160 mg/kg bw

No report submitted. Data taken from review article (Ref 1, subm I)

Species/strain:	Rat, Sprague Dawley
Group size:	5 males and 5 females
Test substance:	"Textile grade indigo dispersible powder"
Dose:	5000 mg/kg bw
Vehicle:	50% aqueous suspension
Results:	no mortalities, LD50orl> 5000 mg/kg bw

No report submitted. Data taken from review article (Ref 1, subm I)

Species/strain:	Rat, Sprague Dawley
Group size:	5 males and 5 females
Test substance:	"Indigo 20% paste, textile grade"
Dose:	5000 mg/kg bw
vehicle:	6% sodium hydroxide solution
Results:	2/5 males and 5/5 females died, mortality was comparable to that in rats receiving 6% sodium hydroxide vehicle and ascribed to the caustic nature of the substrate.

No report submitted. Data taken from review article (Ref 1, subm I)

Species/strain:	Dog, mongrel
Group size:	1 male and 1 female per group
Test substance:	"Certified D&C Blue No.6"
Dose:	31.6, 100, 316 and 1000 mg/kg bw
vehicle:	not stated
Results:	no mortalities

No report submitted. Data taken from review article (Ref 1, subm I)

1.3.2. Acute dermal toxicity

Species/strain:	New Zealand White Rabbits
Group size:	5 males and 5 females
Test substance:	"Textile grade 20% indigo"
Dose:	2000 mg/kg bw
Vehicle:	not stated
Results:	eschar formation at treatment sites; no mortalities, LD50drm> 2000 mg/kg bw

No report submitted. Data taken from review article (Ref 1, subm I)

1.3.3. Acute inhalation toxicity

Species/strain:	Rat, Sprague Dawley
Group size:	5 males and 5 females
Test substance:	aerosol of "20% indigo textile grade"
Time weighted conc.:	0.08 mg/l
Droplet size:	74% < 10 microns (mean size 4.6 + 3.3 microns)
Results:	no mortalities

No report submitted. Data taken from review article (Ref 1, subm I)

Species/strain:	Rat, Sprague Dawley
Group size:	5 males and 5 females
Test substance:	"indigo dispersible powder", not otherwise characterised
Time weighted conc.:	0.76 mg/l
Droplet size:	79% < 10 microns (mean size 2.1 + 2.3 microns)
Results:	bluish discoloration of lungs, no mortalities

No report submitted. Data taken from review article (Ref 1, subm I)

1.3.4. Repeated dose oral toxicity

No data submitted

1.3.5 Repeated dose dermal toxicity

Guideline:	/
Species/strain:	Rabbits
Group size:	1-4 males and females per group
Test substance:	"Certified D&C Blue No.6"
Dose levels:	500 mg/kg bw/day; at 0.1 or 1.0% in the vehicles; 5 days a week
Vehicles:	white petrolatum and hydrophilic ointment
No. applications:	15(3 weeks)or 64 (13 weeks)
Results:	It was stated that the test substance was without effect on skin conditions or microscopic examination of a number of organs. No further details were, however, provided.

No report submitted. Data taken from review article (Ref 1, subm I)

Guideline:	/
Species/strain:	Swiss Webster Mice
Group size:	50 males and 50 females per group
Test substance:	"Certified D&C Blue No.6"
Dose levels:	1 mg per animal at 0.1% in the vehicle; once a week
Vehicle:	benzene
Exposure:	95 weeks
Results:	It was stated that no evidence was found that repeated dermal application produced any test substance related effects. No further details were, however, provided.

No report submitted. Data taken from review article (Ref 1, subm I)

1.3.6. Repeated dose inhalation toxicity

No data submitted

1.3.7. Subchronic oral toxicity

Guideline:	/
Species/strain:	rat, "Charles River albino"
Group size:	10 males per group
Test substance:	"Certified D&C Blue No.6"
Dose levels:	0, 0.1, 0.23, 0.55, 1.29 and 3% in the diet
Exposure:	6 weeks

Results
Clinical signs: No treatment related effects reported
Body weight: /
Food intake: /
Haematology: /
Clin. Chemistry: /
Organ weights: No treatment related effects reported
Macroscopy: Colour retention in fatty tissues, affected groups not stated
Histopathology: Degenerative centrilobular hepatocytic changes in the high-dose group

No report submitted. Data taken from review article (Ref 1, subm I)

1.3.8. Sub-chronic dermal toxicity

No data submitted

1.3.9. Sub-chronic inhalation toxicity

No data submitted

1.3.10. Chronic toxicity

Guideline: /
Species/strain: rat, "Charles River albino"
Group size: 25 males and 25 females per group
Test substance: "Certified D&C Blue No.6"
Dose levels: 0, 0.25, 1.0 and 3.0% in the diet
Exposure: 2 years (interim sacrifice of 5 rats/sex/group after 1 year)

Results
Clinical signs: No treatment related effects
Survival: No treatment related effects
Body weight: /
Food intake: Decreased in the high-dose group during the first 6 months
Haematology: Consistently decreased haematocrit and haemoglobin in high-dose males
Clin. Chemistry: Not conducted
Urinalysis: Bilirubinuria in mid- and high-dose groups at 24 month stage
Macroscopy: No treatment related effects
Microscopy: No treatment related effects (in 10 rats of control and high-dose group both after 1 and 2 years)

No report submitted. Data taken from review article (Ref 1, subm I)

Guideline: /
Species/strain: dog, beagle
Group size: 3 males and 3 females per test group; controls 10 dogs/sex/group
Test substance: "Certified D&C Blue No.6"
Dose levels: 0, 0.25, 1.0 and 3.0% in the diet
Exposure: 2 years

Results
Clinical signs: No treatment related effects
Haematology: No effects were reported
Clin. Chemistry: No effects were reported
Urinalysis: No effects were reported
Organ weights: No treatment related effects

Macroscopy: No treatment related effects
Microscopy: No treatment related effects

No report submitted. Data taken from review article (Ref 1, subm I)

1.4. Irritation & corrosivity

1.4.1. Irritation (skin)

No data submitted

1.4.2. Irritation (mucous membranes)

No data submitted

1.5. Sensitisation

No data submitted

1.6. Teratogenicity

Three generation study

Guideline: /
Species/strain: rat, "Harlan Wistar"
Group size: 10 males and 20 females per group
Test substance: "Certified D&C Blue No.6"
Dose levels: 0, 5, 50 150 and 500 mg/kg diet

Results
It was concluded that no deleterious effects were associated with the inclusion of the test substance in the diet of rats for 3 generations. No further details were, however, provided. No report submitted. Data taken from review article (Ref 1, subm I)

Teratogenicity studies

Guideline: /
Species/strain: rat, "Charles River CD"
Group size: 20 females (mated) per group
Test substance: "Certified D&C Blue No.6"
Dose levels: 50, 160 and 500 mg/kg bw/day
Vehicle: methyl cellulose suspensions
Treatment period: days 6-15 of gestation

Results
It was stated that the test substance was without effect on reproduction performance, maternal weight gain and foetal development. No further details were, however, provided. No report submitted. Data taken from review article (Ref 1, subm I)

Guideline: /
Species/strain: New Zealand White rabbits
Group size: 10 females (mated) per group
Test substance: "Certified D&C Blue No.6"
Dose levels: 50, 160 and 500 mg/kg bw/day
Vehicle: methyl cellulose suspensions

Treatment period: days 6-18 of gestation

Results
It was stated that the test substance was without effect on reproduction performance, maternal weight gain and foetal development. No further details were, however, provided. No report submitted. Data taken from review article (Ref 1, subm I)

1.7. Toxicokinetics (incl. Percutaneous Absorption)

No data submitted

1.8. Mutagenicity/Genotoxicity

1.8.1 Mutagenicity/Genotoxicity *in vitro*

Reverse Mutation Testing using Bacteria

Different batches of synthetic Indigo were tested on TA 98, TA 1535, TA 1537, TA 1538 and TA 100 of *S. typhimurium* in the presence and in the absence of Aroclor 1254 rat liver homogenate at the doses of 0, 30, and 150 µl/plate with TA 98 and TA 100), 0, 0.4, 1.0, 2.5, 4.0 and 10.0 µmol/plate with TA 98 and 0, 0.4, 1.0, 3.8 and 19.1 µmol/plate with TA 1535, TA 1537, TA 1538: the test items were found mutagenic on TA 98 and TA 1538 in the presence of increasing amounts of S9.
In the same conditions, purified synthetic Indigo and natural Indigo (nature not specified) were found non mutagenic.

Ref.: 10 (subm I)

Reverse Mutation Testing using Bacteria

Synthetic Indigo was tested in strains of *Salmonella typhimurium* TA98, TA100, TA1535 and TA1538 in the presence and in the absence of a metabolic activation system. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn. Negative and positive controls were included.
In the absence of S9-mix a biologically relevant increase in the number of revertant colonies was not observed in any of the strains used. In the presence of S9-mix (25 µl/plate) a dose-dependent and statistically significant increase in the number of revertant colonies was observed in strains TA 98 and TA 1538.

Ref.: 11 (subm I)

Reverse Mutation Testing using Bacteria

Synthetic indigo (98 % pure, dissolved in DMSO) was tested at concentrations of 0, 150, 300, 600 and 1200 µg/plate in strains of *Salmonella typhimurium* TA98 and TA100 in the presence and in the absence of a metabolic activation system. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
In the presence but not in the absence of S9-mix a dose-dependent and statistically significant increase in the number of revertant colonies was observed in both strains TA 98 and TA 1538.

Ref.: 12 (subm I)

Reverse Mutation Testing using Bacteria

Indigo (92% pure, dissolved in DMSO)was tested in strains of *Salmonella typhimurium* TA98 in the presence and in the absence of a metabolic activation system. Liver S9 fraction from 3MC-induced rats was used as exogenous metabolic activation system.
In the presence but not in the absence of S9-mix a dose-dependent and statistically significant increase in the number of revertant colonies was observed in strain TA 98

Ref.: 13 (subm I)

1.8.2 Mutagenicity/Genotoxicity *in vivo*

No data submitted

1.9. Carcinogenicity

Oral administration, rat
A 2-year chronic feeding study of C.I. 73000 was carried out with 3 groups of 25 male and 25 female Charles River adult albino rats and a control group of 80 males and 80 females. The test material was incorporated in the basal diet at levels of 0.25, 1.0 and 3.0%. Throughout the 2-year study, observations were made daily for mortality and weekly for gross signs of toxicity.
Autopsies were performed on all animals, which died during the study. At the termination of the study, histopathology was performed on all preserved tissues from 10 male and 10 female rats in the control group and on an equal number in the high dosage group. Appearance and behaviour of the test rats were generally comparable to those of the controls. At the 3% level, food consumption was significantly lower than controls for the first six months, but comparable to controls during the remainder of the study. Autopsies performed on the animals, which died during the second year of the study did not reveal any consistent gross related effects on the kidneys or other tissues in either sex. It is concluded that the study appeared to demonstrate that after a period of adjustment to higher dosage levels, the rats were able to tolerate up to 3% of synthetic indigo in their diet without serious effects (US FDA 1962-1973).

Skin painting, mice
C.I. 73000 was applied as a 1% solution, once per week for up to 95 weeks to 50 male and 50 female Swiss-Webster mice. 100 males and 100 females were used as negative controls. 50 males and 50 females received application of the vehicle (benzene). Autopsies were performed on all sacrificed animals in the absence of marked autolysis. Microscopic examination of the lungs, liver and skin on 10 negative controls, 10 vehicle controls and 10 compound-treated animals were performed at 75 weeks. At the terminal sacrifice (96 weeks), sections of lung liver and skin from 29 negative controls, 26 vehicle controls and 13 compound-treated animals were examined microscopically. Histopathology was also performed on most tissue masses and on grossly abnormal organs of the animals. No evidence was found that repeated dermal applications produced any effects attributable to synthetic indigo lesions. The tumours seen in treated mice were comparable to the tumours in the vehicle controls (US FDA 1962-1973).

Ref.: 1 (subm I)

Human studies

No data submitted

1.10. Special investigations

No data submitted

1.11. Safety evaluation

Not applicable

1.12. Conclusions

Toxicity
Most of the data were derived from a review article (Ref 1) without sufficient details to enable evaluation by the SCCNFP.

Irritation and sensitisation
No data on have been provided on the irritation/sensitisation potential of CI 73000.

Percutaneous absorption
No data on dermal penetration have been provided on CI 73000. A skin permeation study should have been performed with CI 73000.

Mutagenicity/genotoxicity
The test item, as synthetic Indigo, has been tested in different strains of *Salmonella typhimurium* In the presence of S9-mix dose-dependent and statistically significant increases in the number of revertants were reported for strains TA 98 and TA 1538.

Carcinogenicity
It is not possible to make any conclusions regarding carcinogenicity from the data available.

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